Human Cytomegalovirus US28 Found in Glioblastoma Promotes an Invasive and Angiogenic Phenotype

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Abstract

Human cytomegalovirus (HCMV) infections are seen often in glioblastoma multiforme (GBM) tumors, but whether the virus contributes to GBM pathogenesis is unclear. In this study, we explored an oncogenic role for the G-protein–coupled receptor-like protein US28 encoded by HCMV that we found to be expressed widely in human GBMs. Immunohistochemical and reverse transcriptase PCR approaches established that US28 was expressed in approximately 60% of human GBM tissues and primary cultures examined. In either uninfected GBM cells or neural progenitor cells, thought to be the GBM precursor cells, HCMV infection or US28 overexpression was sufficient to promote secretion of biologically active VEGF and to activate multiple cellular kinases that promote glioma growth and invasion, including phosphorylated STAT3 (p-STAT3) and endothelial nitric oxide synthase (e-NOS). Consistent with these findings, US28 overexpression increased primary GBM cell invasion in Matrigel. Notably, this invasive phenotype was further enhanced by exposure to CCL5/RANTES, a US28 ligand, associated with poor patient outcome in GBM. Conversely, RNA interference–mediated knockdown of US28 in human glioma cells persistently infected with HCMV led to an inhibition in VEGF expression and glioma cell invasion in response to CCL5 stimulation. Analysis of clinical GBM specimens further revealed that US28 colocalized in situ with several markers of angiogenesis and inflammation, including VEGF, p-STAT3, COX2, and e-NOS. Taken together, our results indicate that US28 expression from HCMV contributes to GBM pathogenesis by inducing an invasive, angiogenic phenotype. In addition, these findings argue that US28–CCL5 paracrine signaling may contribute to glioma progression and suggest that targeting US28 may provide therapeutic benefits in GBM treatment. Cancer Res; 71(21): 6643–53. ©2011 AACR.

Introduction

Human cytomegalovirus (HCMV) is a beta-herpesvirus that can cause life-threatening infections in human fetuses and immunocompromised individuals. It is a major cause of congenital brain infection and disability in humans. Our laboratory first reported the association of HCMV infection with human glioblastomas (GBM; ref. 1). These findings have been confirmed by other groups (2), and clinical trials are currently underway for the treatment of GBM with anti-HCMV agents and immunotherapy approaches. A growing body of evidence suggests that HCMV infection of malignant glioma does not simply represent an epiphenomenon but rather expression of HCMV gene products in tumor cells and the tumor microenvironment directly impacts tumor progression. Several HCMV gene products have been found to have mutagenic and transforming potential (3). We previously showed that the HCMV IE1 gene product promotes GBM mitogenicity by interfering with Rb, p53, and AKT signaling pathways (4) and that HCMV envelope glycoprotein B can directly activate oncogenic signaling pathways through activation of the PDGFR-α receptor tyrosine kinase (5).

US28 is an HCMV-encoded G-protein–coupled receptor that is a homologue of the human CCR1 chemokine receptor. US28 is constitutively active and may be further activated by binding of several ligands: SDF-1, CCL2/MCP-1, CCL5/RANTES, and CX3CL1/Fraktalkine (6). US28 has properties of a viral onco-gene, because ectopic expression of US28 can induce a proangiogenic and transformed phenotype in vivo via activation of the NF-κB and COX2 signaling pathways (7). A recent report showed that US28 induces interleukin-6 (IL-6) and VEGF through NF-κB activation, resulting in potent activation of the STAT-3 transcriptional activator in NIH 3T3 mouse fibroblasts (8).

We sought to determine whether US28 could (i) influence early events that lead to gliomagenesis in neural precursor cells and (ii) promote key oncogenic features in established...
Glioblastoma cells. We hypothesized that US28 expression in neural precursor cells (NPC), possibly the glioma "cell of origin", may promote the pathogenesis of GBM. We evaluated changes that occur in gene expression patterns and in the angiogenic and invasion pathways in adult NPCs, when infected by HCMV or overexpressing US28. Next, we assessed the changes in the invasive and angiogenic phenotypes of primary GBM cultures induced by US28 overexpression. Finally, we conducted loss-of-function studies in human GBMs persistently infected with HCMV to show the specificity of US28-induced pathogenesis.

Materials and Methods

Cell culture

U251 and U87 cell lines were obtained from American Type Culture Collection (ATCC) and grown in DMEM/Ham's F-12 + 10% FBS. Primary glioblastoma/neural precursor cell-derived cultures were generated with tissue from surgical resections at the California Pacific Medical Center obtained according to the Institutional Review Board-approved protocol. Tissues were dissociated by enzymatic and mechanical dissociation as previously described. Single-cell suspensions were cultured with neural basal medium + N2 supplement, 20 ng/mL epidermal growth factor (EGF), 20 ng/mL basic fibroblast growth factor, and 1 μg/mL laminin as previously described (9). For ELISA for VEGF experiments and tube formation assays, cells were cultured in the absence of FBS or growth factors at least 48 hours prior to media collection. The NPC cell line was derived from the hippocampus tissue removed from a patient with intractable epilepsy. Cells were characterized by immunofluorescence and found positive for Nestin, GFAP, TuJ1, and Olig2. All experiments were carried out on passages 2 to 5 from the NPC culture. Human umbilical vein endothelial cells (HUVEC) were obtained from Invitrogen and grown in the complete endothelial cell growth media recommended by the manufacturer.

US28 expression vectors

The Ad-US28 and Ad-Control adenoviruses were a gift from Dr. Dan Streblow, Oregon Health & Science University. The pcDEF-US28 plasmid was a gift from Dr. Martine Smit. The US28 insert was excised from the pcDEF plasmid and cloned into the pLXSN vector (4). Retroviruses were produced and used to infect glioma cells as previously described by our laboratory (4).

Viruses

The Towne and AD169 HCMV strains were obtained from ATCC and grown in human embryonic fibroblasts, as previously described (10). The TR virus strain was a gift from Dr. Lee Fortunato, University of Idaho (Moscow, ID).

Knockdown experiments using siRNA to US28

US28 knockdown was achieved with 2 siRNA oligonucleotide duplexes custom synthesized by Dharmacon. The sense sequences for the 2 siRNAs are as follows: CGACGGAGUUUGA-CUACGAUU(1) and CUCACAAUUACCGUAUU(2). Experiments were carried out with each siRNA individually and the 2 duplexes were combined. As a negative control, nontargeting control pool from Dharmacon (D-001810-10-05) was used. Effective protein knockdown was verified at 48 and 72 hours post-transfection and prior to functional assays, as described later.

Fluorescence measurements to quantify US28 expression levels

Images were taken at fixed exposure times with an Axio Image Z2 microscope (Zeiss). The fluorescence intensities, from at least 100 cells, were quantified with ImageJ software; plots representing cumulative distribution of mean pixel intensity for various conditions are shown. The Kolmogorov–Smirnov test was used to determine whether the measured differences were statistically significant.

Expression profiling using the HCMV DNA array and the Affymetrix Gene ST1 array

Total RNA was isolated and the quality verified as described later for reverse transcriptase PCR (RT-PCR). The RNA was processed for microarray hybridization at the Center for Applied Genomics, UMDNJ-New Jersey Medical School. The HCMV arrays were printed and processed as described previously (11). Briefly, the array contains 65-mer oligonucleotides representing 19 predicted open reading frames (ORF) of the HCMV strain AD169, 19 oligonucleotides for ORFs in the Toledo strain that are not found in AD169, and 44 human genes as controls. Total RNA (3 μg) was reverse transcribed to cDNA using SuperScript II RT in the presence of cyanine-3 (Cy3) or cyanine-5 (Cy5) dUTP. The labeled cDNA was purified and hybridized to the arrays at 58°C for 16 hours. The slides were scanned with an Axon 4200AL scanner, and the images were processed with GenePix Pro 6.1. A normalization factor was calculated using 36 human control genes (11) by dividing the median intensity of the Cy5 signal by the median intensity of Cy3 signal of the controls. The data were normalized by multiplying the Cy3 signal of each spot by the normalization factor. The ratio of the Cy5 median intensity to the Cy3 median intensity was determined for each spot and the average ratio determined for the replicate spots. The accession number for data from both Affymetrix and HCMV platforms is GSE31142.

HUVEC tube formation assay

Geltrex (Invitrogen #12760-013) was obtained from Invitrogen and thawed overnight at 4°C. One hundred microliters of Geltrex per well was placed on the bottom of 24-well culture dishes and allowed to solidify at 37°C for 30 minutes. HUVECs were detached with EDTA and resuspended in endothelial cell medium supplemented with various growth factors or conditioned media at 40,000 cells/200μL per well. Tubes were allowed to form for 8 to 10 hours, and cells were visualized with a Nikon Inverted Eclipse TE-2000E microscope, fitted with a CCD Cascade II camera. NIS Elements AR3.0 was used to acquire images, which were further processed in Photoshop.

Statistical data analysis

Significant differences were determined by ANOVA or the unpaired Student t test, where suitable. Bonferroni–Dunn post
hoc analyses were conducted when appropriate. The values of $P < 0.05$ defined statistical significance.

Additional methods are available as Supplementary Information.

**Results**

**US28 is endogenously expressed in human GBMs**

US28 protein expression in human glioblastomas was assessed by immunofluorescence analysis of primary glioblastoma-derived cultures and immunohistochemical analysis of paraffin-embedded tissues from several GBM specimens, including some that were used to generate the primary cultures. reverse transcriptase PCR for US28, HCMV UL56 (a DNA packaging essential viral gene), and Rab14 (human housekeeping gene) was done using RNA isolated from snap-frozen tissues from the same cases. Figure 1A shows an example of immunofluorescence analysis of primary GBM cells that exhibit cytoplasmic and membrane staining for the US28 antigen. Preincubation of the primary antibody with excess US28 blocking peptide showed specificity of immunostaining (Fig. 1B).

As shown in Fig. 1C, US28 expression was detected in paraffin-embedded GBM biopsy specimens. Figure 1D shows specificity of staining, using the US28 blocking peptide in excess, as described earlier. Sections from the same sample show abundant staining for VEGF (Fig. 1E) and COX2 (Fig. 1F), suggesting the presence of enhanced angiogenesis and inflammation in and around the US28-positive tumor cells. Colocalization of US28 and VEGF in another case of primary glioblastoma is shown in Supplementary Fig. S1. The specificity of the US28 antibody was established by comparing immunostaining of cells that were mock-infected, HCMV-infected, or ectopically expressing US28 (Supplementary Fig. S2). To confirm that HCMV US28 mRNA was likewise...
expressed in human GBM specimens, we carried out reverse transcriptase PCR on RNA extracted from GBM biopsy specimens from several different patients. Uninfected NPCs showed no evidence of the amplified US28 gene product, or another conserved HCMV gene product UL56 (Fig. 1G). In contrast, we detected amplified US28 RNA transcripts in the primary GBM biopsy specimens from several patients, including a case found positive by immunohistochemistry (shown in Fig. 1C–F). All amplified US28 reverse transcriptase PCR products were sequenced to confirm specificity to HCMV, and unique gene polymorphisms were identified in several specimens, indicating that no cross-contamination of laboratory or PCR specimen occurred (C-terminal sequences alignment is provided in the Supplementary Information). Additional GBM and control brain tissues were immunostained for US28, COX2, VEGF, phospho-STAT3 (p-STAT3), and endothelial nitric oxide (e-NOS; Supplementary Table S1). Of the 35 different brain tissues screened, 53% were positive for US28 by reverse transcriptase PCR and 65% were positive by immunohistochemistry; there was more than 90% concordance in the results showing US28 detection when both approaches were used (Supplementary Table S1).

**HCMV infection of NPCs induces expression of US28 and CCL5, which together promote glioma invasiveness**

To understand the role HCMV US28 might play in gliogenesis, we first wished to ascertain that US28 is expressed during HMCV infection of human adult NPCs, the purported cells of origin of adult GBM. NPCs were infected with HCMV [Towne and TR strains; multiplicity of infection (MOI) = 1] or mock infected. Total RNA was harvested at 72 hours, and HCMV gene expression was assayed with a custom-made oligonucleotide microarray representing all the predicted ORFs for HCMV (11). The same samples were profiled with human Affymetrix DNA arrays. As shown in Fig. 2A, US28 was among the most abundantly expressed HCMV transcripts following infection with either viral strain. Interestingly, one of most upregulated human transcripts was the chemokine CCL5/RANTES (Fig. 2B, arrow). Although US28 can act as a constitutively active receptor, CCL5 is a bona fide ligand for US28 and can further stimulate US28 signaling, suggesting that US28 and CCL5/RANTES coexpression might induce a potent autocrine signaling loop. To determine whether expression of CCL5 is a relevant biomarker for GBM, we analyzed the REMBRANDT GBM database. We determined that CCL5 expression levels were inversely correlated with survival in human glioblastomas (Fig. 2C). Analysis of previously characterized glioblastoma molecular subclasses (12) showed that CCL5 expression levels are elevated in the “mesenchymal” GBMs, characterized by poor patient outcome (ref. 12; Supplementary Fig. S3).

To assess the effects of US28 expression on glioma invasiveness, we carried out Matrigel invasion assays comparing LXSN with US28-LXSN—transduced U251 and U87 glioma cells and 2 primary glioma cultures, which had no detectable HCMV transcripts. US28 overexpression resulted in an approximately 30% increase in the invasiveness of all glioma cell lines tested (Fig. 2D). The presence of 50 ng/mL recombinant human CCL5 in the bottom chamber further enhanced invasiveness of glioma cells and primary GBM cultures by 50% to 60%, as shown in Fig. 2D. These data show that CCL5, which is upregulated by HCMV infection, can augment US28-induced glioma cell invasion.

To establish the specificity of US28 effects on glioma cell invasion, we used a siRNA approach to knockdown US28 expression in a well-characterized human glioma cell line, U87 (13), persistently infected with HCMV (Supplementary Fig. S4). US28 protein levels were measured by fluorescence intensity measurements of cells processed for US28 immunofluorescence. US28 siRNA1 induced an approximately 40% US28 knockdown, whereas siRNA2 induced approximately 60% US28 knockdown (Supplementary Fig. S5). When used together, siRNA1+2 induced approximately 80% US28 knockdown (Supplementary Fig. S5). We used a CCL5-neutralizing antibody to distinguish between US28 constitutive activity and the response to the CCL5 ligand secreted by human glioma cells. Figure 2E shows that CCL5 levels were significantly (~75%) inhibited in U87 cells by preincubation with a CCL5-neutralizing antibody (20 ng/mL, 12 hours), regardless of the presence of HCMV or US28. Although US28 KD had no effect in uninfected U87 cells, Matrigel invasion of HCMV-positive U87 cells was inhibited by approximately 20% by US28 siRNA1 or 2 used alone and by 30% when the 2 siRNAs were used together (Fig. 2F and Supplementary Fig. S5). Pretreatment with CCL5-neutralizing antibody inhibited glioma cell invasion by approximately 30% to 35% and the use of both US28 knockdown and CCL5 neutralization did not further increase this effect (Fig. 2F and Supplementary Fig. S5). US28 knockdown a primary GBM culture, confirmed to be HCMV positive, resulted in inhibition of tumor cell invasion by approximately 35%, both baseline and in response to CCL5 stimulation (Supplementary Fig. S6).

**US28 activates multiple oncogenic pathways in human NPCs**

To determine additional oncogenic pathways activated by HCMV infection/US28 expression in NPCs, we used a phosphor-kinase human array (R&D Systems) embedded with antibodies specific for multiple phosphoproteins (Fig. 3A and B). Pathways associated with glioma progression and invasion, including p-STAT3, AKT, ERK1/2, FAK, Src, and eNOS, were significantly activated by both whole virus infection and US28 overexpression in NPCs (Fig. 3C). Immunofluorescence analyses of US28 overexpressing NPCs confirmed upregulation of COX2, VEGF, p-STAT3, and e-NOS (Fig. 3D). e-NOS levels, which are elevated in gliomas, correlate with increased tumor aggressiveness (14, 15). In addition to its proangiogenic role, e-NOS mediates production of nitric oxide, which was shown to induce the growth of glioma-initiating cells (16). This is the first report documenting that HCMV US28 induces e-NOS activation, which contributes to glioma pathogenesis.

Using Western blotting and immunofluorescence, we confirmed that US28 induces p-STAT3 in neural precursor cells (Supplementary Fig. S7). STAT3 activation is critical for NPC malignant transformation toward a mesenchymal GBM.
phenotype (17), suggesting that US28-induced activation of p-STAT3 may contribute to gliomagenesis. Consistent with a recent report, we also found that US28 and p-STAT3 colocalize in primary glioblastomas in situ (Supplementary Fig. S8), which would explain why HCMV-positive glioma cells exhibit activation of the STAT3 pathway, implicated in promoting immunosuppression, maintenance of glioma stem cells, and tumor progression (8, 18).

US28 promotes GBM angiogenesis

We next investigated whether US28 can modulate VEGF levels in neural precursor and glioma cells by immunofluorescence and ELISA. Figure 4A shows that VEGF is significantly upregulated in US28-expressing NPCs. VEGF levels were measured in 4 different cell types (NPC, U251 and U87 glioma cell lines, and a primary GBM–derived line) by a highly sensitive ELISA. Seventy-two hours following infection with either Towne or TR HCMV strain, or US28 overexpression, VEGF was induced more than 2-fold in all cell types tested (Fig. 4B). US28 overexpression alone was sufficient to induce equivalent levels of VEGF expression to those found after whole HCMV infection, suggesting that US28 may play a predominant role in the HCMV-induced VEGF secretion. Remarkably, NPCs, which are
nonmalignant, were also induced to produce VEGF, suggesting that US28 expression may promote an angiogenic phenotype in normal adult neural cells. An HUVEC tube formation assay was used to quantify angiogenesis. Figure 4C shows that NPC HCMV–infected or overexpressing US28 produced supernatant enriched in proangiogenic growth factors that induced a dramatic increase in HUVEC tube formation compared with mock infection or transduction with control vector (Fig. 4D). These data indicate that US28 expression in a normal neural precursor cell could stimulate angiogenesis of neighboring endothelial cells. To show specificity of the US28 proangiogenic activity, we carried out

Figure 3. US28 induces activation of cellular kinases involved in glioma pathogenesis. A and B, HCMV (Towne; MOI = 1) and mock-treated NPCs (A) and glioma cells (B) were profiled with a phosphor-kinase human antibody array. C, densitometry measurements were done per the manufacturer’s instructions. Percentage of change in phosphorylation levels between HCMV/US28-treated and control cells is shown. One (of 2) representative experiment is shown. D, double immunofluorescence for US28 and the indicated proteins in NPCs transduced with LXSN-US28 for 48 hours. Right, IgG staining controls. Nuclei were counterstained with propidium iodide. Bar, 50 μm.
Figure 4. US28 promotes glioma angiogenesis. A, NPCs transduced with either LXSN-HA-US28 or Ad-US28 and control LXSN/mock-treated cells were processed for immunofluorescence. Right, NPCs that express US28 (green) secrete VEGF (blue), as shown by colocalization of the 2 markers. Nuclei are stained with propidium iodide. Bar, 100 μm. B, NPC, U251, U87, and a primary GBM line (4121) were treated with HCMV (Towne and TR; MOI = 1), transduced with Ad-US28, or treated with EGF (50 ng/mL) in serum-free media. Supernatants were used in an ELISA for VEGF. Samples were assayed in quadruplicate, and the experiment was repeated twice. Comparisons between treated and mock within the same cell line were analyzed by ANOVA. *, P = 0.02; **, P < 0.002. C, NPC-derived supernatants were tested in HUVEC tube formation assays. Complete endothelial cell growth media was used as a positive control. Representative photomicrographs are shown. Each condition was assayed in 6 wells of a 24-well plate, and the experiment was repeated twice. Bar, 100 μm. D, average numbers of branch points and endothelial cell lumens are shown from 1 representative experiment. Comparisons were analyzed by ANOVA. **, P < 0.02 in all cases.
US28 knockdown in HCMV-infected glioma cells inhibits VEGF secretion and subsequent angiogenesis. A, immunofluorescence was used for detection of US28 (green) and VEGF (red) in U87 cells persistently infected with HCMV treated either with control siRNA (top) or siRNA1–2 targeting US28. B, cumulative distribution of mean pixel intensity per cell obtained from immunofluorescence detection of US28 and VEGF in U87 cells treated with either targeting (siRNA1–2) or control siRNAs. The Kolmogorov–Smirnov test was used to determine significance of differences in the fluorescence intensity measured in more than 100 cells per condition, *P = 0.001; **P = 0.002. C, VEGF levels were measured by ELISA in U87 glioma cells and primary 4121 GBM cells uninfected or HCMV-infected in the presence of either control or US28 targeting siRNA1, siRNA2, or siRNA1–2. Differences were significant, *P = 0.05; **P = 0.002, the Student t test. D, quantification of HUVEC branches and lumens formed in each of the indicated conditions. ***, P < 0.02, ANOVA. E, representative photomicrographs of HUVEC tube formation assay in the presence of various types of conditioned media, as indicated. Bar, 100 μm. HUVEC tube formation assays were repeated 3 times, each condition was run in quadruplicate.

loss-of-function experiments, using siRNA to knock down US28 in persistently infected glioma lines. US28 knockdown inhibited VEGF production and glioma cell–mediated angiogenesis as measured by HUVEC tube formation assays. Figure 5A illustrates US28 and VEGF detection in persistently infected U87 glioma cells before and after US28 knockdown. We used quantification of immunofluorescence signals to measure the extent of US28 protein knockdown (Fig. 5B and Supplementary Fig. S5). Cumulative distribution of pixel intensity for immunopositivity illustrates that approximately 80% of US28-positive cells lost their signal after treatment with targeting US28 siRNA1–2, confirming effective protein knockdown (Fig. 5B). A similar level of US28 knockdown was achieved in the 4121-HCMV–infected cells following 72-hour treatment with targeting siRNA1–2 (data not shown). VEGF secretion was inhibited by US28 knockdown (Fig. 5B, bottom). Using ELISA, we determined that VEGF levels (initially induced by HCMV) were inhibited by 35% in HCMV-infected U87 and primary glioma cells, following US28 knockdown using siRNA1–2 (Fig. 5C). Each US28 siRNA used separately had a more modest effect in inhibiting VEGF secretion, whereas uninfected glioma cells did not show a change in VEGF levels, confirming specificity of the US28 knockdown effect (Fig. 5C). Supernatants from persistently infected glioma cells with or without US28 siRNA1–2 were used in an HUVEC tube formation assay. As shown in Fig. 5D and E, US28 knockdown significantly inhibited the proangiogenic activities of the HCMV-positive glioma cell supernatants. US28 knockdown in an endogenously infected primary GBM-derived culture inhibited VEGF secretion by approximately 50% (Supplementary Fig. S6), suggesting potential therapeutic benefits for targeting US28 in GBM patients.

Further analysis of primary GBM cells from patients identified several tumor cases in which US28 expression was significant and where VEGF expression had a high level of colocalization with US28 (Fig. 6A–C and Supplementary Table S1). Immunofluorescence analysis of primary GBM cells for eNOS and US28 indicated that US28 also colocalized with eNOS (Fig. 6D–F). Using paraffin-embedded tissue samples from the same patient, we found HMCV US28, VEGF, e-NOS, and COX2 coexpressed both in tumor cells and within the tumor
Figure 6. HCMV US28 colocalizes with markers of invasiveness and angiogenesis in situ. A–F, primary glioblastoma-derived cells were processed for immunofluorescence with antibodies against US28 (A and D), VEGF (B), and e-NOS (E). C and F, merged photomicrographs of colocalization of US28 and the 2 markers of angiogenesis. Nuclei are counterstained with propidium iodide. Bar, 100 μm. G–L, consecutive paraffin sections (5 μm apart) from a glioblastoma specimen were stained for US28, VEGF, e-NOS, and COX2 and developed with horseradish peroxidase-3,3′-diaminobenzidine. Arrows indicate cells positive for several markers in the same area. Counterstaining, hematoxylin. Bar, 50 μm. M, summary of the autocrine and paracrine signaling pathways through which US28 promotes GBM growth, invasion, and angiogenesis.
microenvironment (Fig. 6G–I), suggesting that proinflammatory and proangiogenic signaling is, at least in part, initiated and promoted by US28 expression in infected GBM cells. Together with the other already described mechanisms, such as activation of the IL-6–p-STAT3 pathway (8), and induction of CCL5 (our data), HCMV US28 emerges as a key regulator of GBM progression by enhancing tumor cell invasion and angiogenesis (Fig. 6M).

Discussion

Our laboratory first identified and reported the presence of various HCMV proteins in human glioblastoma. In this study, we investigated the role of US28 in driving critical signaling pathways supporting glioma growth such as invasion and angiogenesis.

We carried out a systematic screening of human primary glioma tissues and controls by immunohistochemical and reverse transcriptase PCR/sequencing, which indicate that approximately 60% of human GBMs are US28 positive by one or more techniques. On the basis of these findings, we hypothesized that US28 expression in normal neural precursor cells might promote gliomagenesis and that US28 expression in established GBM cells could promote tumor angiogenesis and invasion.

The experimental findings we present here support our hypothesis. When we infected NPCs with either a laboratory or clinical HCMV isolate, we detected US28 among the most highly expressed HCMV genes. Furthermore, HCMV infection of NPCs resulted in approximately 40-fold increase in CCL5 mRNA levels, which could further enhance US28-mediated signaling in an autocrine manner because CCL5 binds and activates US28 (19). CCL5 overexpression has been previously associated with glioblastoma (20), and we determined that its expression levels correlate with poor GBM patient outcome by interrogating a public database.

Our data show for the first time the existence of an autocrine signaling loop in HCMV-infected or US28-expressing glioma cells that respond to CCL5 stimulation with increased invasive behavior. Interestingly, this interaction seems to be cell-type specific, as another study has shown that macrophages expressing US28 migrate toward Fraktalkine (another US28 ligand) rather than CCL5 (21). In the context of glioma-associated inflammation, US28 may therefore modulate multiple autocrine and paracrine loops, promoting an oncogenic tumor microenvironment. US28 overexpression also induced approximately 3-fold increase in VEGF levels in both NPCs and GBM cells. Together, these data indicate that US28 induces a proangiogenic and invasive phenotype both in malignant glioma cells and in NPCs. To ascertain specificity of the proinvasive and proangiogenic activities of US28, we carried out loss-of-function experiments in persistently infected glioma cells and a primary glioma culture. We used the US7 glioma cell line bearing well-defined genomic alterations in conjunction with 2 custom-made siRNA duplexes used alone and in combination to assess the specificity of US28 effects. Our data show that US28 knockdown significantly inhibited GBM cell invasion and secretion of biologically active VEGF in HCMV-positive gliomas. These results also suggest that targeting US28 may have therapeutic benefits for GBM patients.

Immunohistochemical analysis of biopsy specimens of patients with glioma showing colocalization of US28 with VEGF, COX2, p-STAT3, and e-NOS in situ suggests once again that multiple US28-driven mechanisms contributing to the aggressiveness of primary GBMs may exist. Our data add to and corroborate recent reports indicating that HCMV US28 expression can promote oncogenesis. Maussang and colleagues have shown that US28 expression can induce oncogenic transformation of 3T3 fibroblasts, and this work was recently extended upon by the demonstration that a critical mediator or the US28 oncogenic signaling pathway is NF-κB, whose activation drives expression of VEGF and COX2 (22). Our data indicate that biologically active VEGF is induced by US28 in neural precursors and glioma cells and that COX2 and VEGF are coexpressed with US28 in GBMs.

Slinger and colleagues recently showed that US28 expression could be detected in human GBM specimens and that it activates the IL-6–STAT3 signaling pathway in a fibroblast model system (8). Our data show for the first time that US28 induces p-STAT3 activation in NPCs and documents colocalization of US28 and p-STAT3 in primary GBM cultures. A recent report showed that expression of HCMV US28 in intestinal epithelial cell lines led to high penetration of adenocarcinomas in a transgenic mouse model of intestinal neoplasia (23). These tumors could be accelerated by coexpression of an US28 ligand, CCL2. Consistent with these observations, our results show that US28 increased glioma cell invasion, which was further enhanced by the addition of another US28 ligand, CCL5, a proinflammatory cytokine associated with aggressive GBMs.

Taken together, our data suggest that HCMV US28 may be a critical factor in promoting the transformation of NPCs and the invasive and angiogenic properties of established glioblastomas. Our results suggest that targeting specific HCMV proteins (e.g., US28) in endogenously infected GBMs may disrupt critical pathways and constitute a novel antitumor approach.

Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interest.

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